Use of Alizarin Complexone Immersion for Marking Otoliths of Mozambique Tilapia

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Abstract: We analyzed the efficacy of alizarin complexone (AC) immersion for creating visible fluorescent marks on otoliths of Mozambique tilapia (*Tilapia mossambica*) and compared the success of processing the otoliths as whole mounts and sections. We immersed 51 tilapia in a buffered 21 C aerated bath of 100 mg/liter AC for 15 hours, and no mortality resulted from marking procedures. Otoliths were removed on days 1, 10, 20, 30, and 60 following marking. Whole ground mounts showed visible rings only 84% of the time, many of which were faint or incomplete. Sectioning revealed visible rings 98% of the time and appeared to be the better examination technique. There was no mark loss over time, and marks on whole ground otoliths became more visible with time (day 1–20 had 60% visibility; day 30–60 had 95% visibility) which was believed to be a result of mark proximity to otolith edge. We concluded that AC could be effectively used to mark Mozambique tilapia otoliths, and suggest sectioning be used for improved mark identification for short-term studies.

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Daily otolith rings have been widely used to estimate the ages of many species of age-0 fishes (Jones 1986), providing valuable information on early life history and factors affecting recruitment (Jones 1992). Daily age information is essential for determination of hatching times, growth, and mortality rates in young fish (Miller and Storck 1984, Essig and Cole 1986, Graham and Orth 1987, Isely and Noble 1987, Pepin 1989, Jones 1992). It is necessary, however, to validate the temporal periodicity of otolith increment formation because not all species deposit rings daily (Geffen 1982), and increment formation may not occur at the same time in different species (Jones 1986).

One common method of validation applied to daily otolith ring formation is the use of chemical marks applied by group marking (Blom et al. 1994). This mark serves as a reference point for establishing the periodicity of increment formation

following mark incorporation. Oxytetracycline (OTC) has been widely used to mark fish by immersion (Weber and Ridgeway 1962, Campana and Neilson 1982, Tsukamoto 1985, Dabrowski and Tsukamoto 1986, Sweatman and Kohler 1991), injection (Weber and Ridgeway 1962, Campana and Neilson, 1982, McFarlane and Beamish 1987), or oral administration (Weber and Ridgeway 1962, 1967; Pedersen and Carlsen 1991; Nordeide et al. 1992). OTC has an antibiotic effect, however, and could have undesirable effects due to the spreading of antibiotics (Jacobsen and Berglind 1988, Grave et al. 1990, Pouliquen et al. 1992). As alternatives to OTC, various other chemicals have been administered by immersion to early life stages of fish. These include alizarin complexone (AC) (Blom et al. 1994, Ahrenholz et al. 1995, Beckman and Schulz 1996, Fitzhugh et al. 1997), which is the most widespread chemical used for immersion marking after OTC (Blom et al. 1994).

In tropical systems, ageing of fish using otoliths has enjoyed variable success. Adult largemouth bass (*Micropterus salmoides*) fail to produce annual growth rings (Neal et al. 1997), but daily growth rings have been validated and are widely used (Churchill et al. 1995). Use of daily rings for cichlids, however, has proven more difficult. The goal of this study was to determine the efficacy of AC immersion for marking otoliths of Mozambique tilapia for use in future age validation studies on this and other cichlid species. The emphasis of the study was on mortality due to a marking procedure, marking success, and retention of otolith marks. We also compared 2 different techniques of otolith processing for frequency of mark detection. Our methods did not include prolonged exposure to a natural photoperiod, and thus we did not attempt to validate the formation of daily rings.

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Methods

Tilapia were obtained from a private producer and ranged 40–56 mm in total length with a mean weight of 1.80 g. Tilapia were placed in a 56-liter aquarium and fed formulated dry feed once per day (approximately 2% body weight) prior to marking. We immersed 51 tilapia in a 21 C aerated bath of 100 mg/liter AC (Alizarin-3-methylamine-N, N-diacetic acid, $C_{19}H_{15}NO_8$) buffered with sodium bicarbonate to pH = 7. After immersion for 15 hours, the fish were transferred to untreated water to rinse away residual AC before placing them back into the aquarium. We continued feeding 2% body weight of dry feed per day, and temperature fluctuated between 20.5 and 25.5 C. Any mortalities were removed and recorded during the marking and growth periods.

Tilapia were sacrificed for otolith removal and analysis beginning 24 hours after immersion (day 1). Subsequent samples were collected on days 10, 20, 30, and 60.

Ten fish were chosen randomly in each sample, and total length (mm) and weight (g) were recorded before removal of both sagittae. Only 9 fish remained for the day-60 sample. These data were used to calculate mean growth rate (g/day) using linear regression on weight over time since marking.

Otoliths were stored dry until preparation, where right or left sagittae were randomly selected and affixed to a glass microscope slide using thermaplastic adhesive before being ground and polished to the primordia on 1 side. Otoliths were viewed using a compound microscope with reflected fluorescent light at 50×–200×.

Otoliths were examined for the presence of an AC mark, and marks were designated as obvious, faint, very faint, or none depending on the visibility of the mark. Otoliths with obvious or faint marks were considered to have visible marks, while otoliths with very faint or no mark were considered to be not visibly marked. Whole ground otoliths that did not show visible marks were sectioned and polished following the techniques of Secor et al. (1992) and the sections were examined for marks using protocols outlined above. We assumed marks that were visible on whole mounts would also be discernible using sectioning. We compared growth rates of fish with and without visible marks using linear regression. For otolith terminology we followed Campana (1992).

Results and Discussion

No mortality occurred during the marking period, and all fish appeared active and healthy following return to the holding aquarium. Tilapia consumed food pellets readily and increased in weight between each removal period (Fig. 1), suggesting that stresses associated with marking may have had minimal effect on fish behavior. Linear regression estimated a growth rate of 0.045 g/day, which was comparable to estimated growth rates (0.054 g/day) for similar size Mozambique tilapia in a Puerto Rico reservoir (R. Noble, unpubl. data). Only 2 unscheduled mortalities occurred

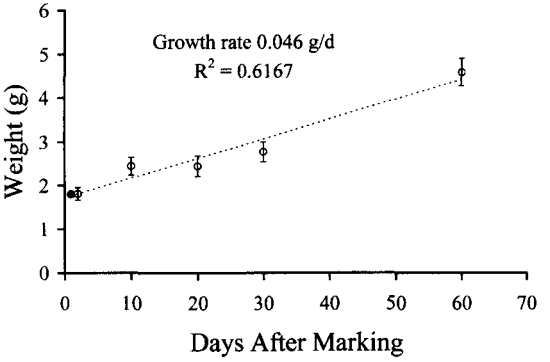

Figure 1. Growth of Mozambique tilapia following immersion in alizarin complexone bath (day 0). Closed circle is the mean weight at marking and open circles are mean weights of tilapia removed during each sample period. Error bars represent SE.

Table 1. Visibility of alizarin complexone mark on whole ground otoliths of Mozambique tilapia viewed under fluorescent microscopy. Marks were designated as obvious, faint, very faint, or none, and only obvious and faint marks were considered visible.

		Mark visibility				
		Visible		Not visible		
Mark age (days)	*N*	Obvious	Faint	Very faint	None	% visible
1	10	6	2	0	2	80
10	10	5	1	1	3	60
20	10	4	0	5	1	40
30	10	8	2	0	0	100
60	9	7	1	1	0	80
Total	49	30	6	7	6	73.5

during the study, which were discovered on days 16 and 18 and appeared unrelated to the AC marking.

The ability to discern marks on the whole ground otoliths (Table 1) was not as high as expected. Over 26% exhibited no visible mark or only traces of AC using the whole otolith examination technique. Only 61% showed easily recognized marks, and many did not exhibit this mark throughout the entire circumference of the otolith. The remaining 6 otoliths (13%) displayed faint marking that was visible only after thorough examination. Growth rates were similar between fish with and without visible marks (Fig. 2). Linear regression estimated growth of fish displaying visible marks at 0.039 g/day, and 0.047 g/day for tilapia not showing visible marks on whole otoliths. This suggests that inability to detect marks was not related to growth since AC uptake is directly related to growth rate.

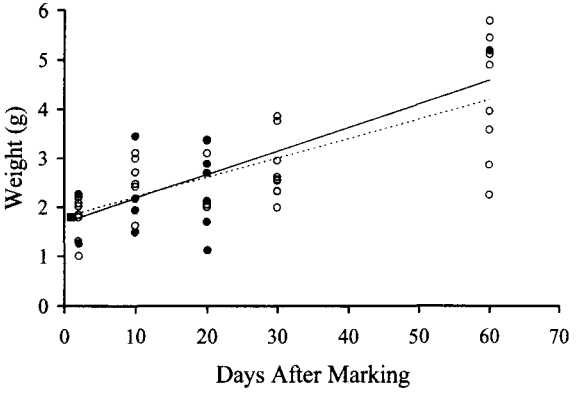

Figure 2. Individual growth of Mozambique tilapia with (open circles) and without (solid circles) visible otolith AC marks. Mean weight at marking (day 0) is indicated by the solid rectangle. Regression lines are associated with visibly marked fish (dotted line) and fish without visible marks (solid line).

Sectioning of otoliths revealed visible fluorescent marks for all but 1 fish from the day-1 sample. Although whole otoliths that contained visible marks were not sectioned, the success of sectioning at revealing previously unseen marks indicates that any mark visible on whole mounts should be visible with sectioning (thus 98% visibility expected from sectioning). The difficulty of detecting marks on otoliths removed 1 day after marking may have been due to insufficient time for mark incorporation, or due to mark location. Recently formed marks would be closer to the otolith edge and more likely to be overlooked as lighting or edge effects. For this reason, growth rates can substantially affect the time required before mark detection is possible, as well as affect the quantity of AC incorporated into the otolith. Although growth rates were comparable to wild fish in this study, inability to detect marks soon after marking suggests a waiting period is appropriate before fish are sacrificed for mark verification.

There did not appear to be mark loss over time; in fact, mark visibility may have increased with time on whole otoliths. Marks were obvious in 79% of otoliths removed on days 30 and 60, with an additional 16% showing faint marks (95% visibility). Only 60% of otoliths from days 1, 19, and 20 displayed visible marks. The reason for this may be that additional otolith growth after marking places the AC mark further from the edge making it easier to differentiate from background lighting and edge effects. Also, these otoliths were moderately cup-shaped with the concave side mounted face up. The process of sanding and polishing may have ground off the outer rings (including the AC mark). This raises additional concern about studies that employ the whole otolith mounting technique.

The inability to discern AC marks on whole ground otoliths from day 1–20 prompts concern that this technique may also overlook outermost daily growth rings due to similar problems (primarily rings lost to grinding). Thus, researchers using whole otolith mounts for daily ageing may want to consider validation procedures to compare ages obtained from each method to ensure accuracy. Since the process of grinding concave otoliths may remove outer rings, a modified technique may be appropriate. The outer portion can be examined and rings counted to a reference point before grinding, then the otolith can be ground to elucidate inner rings which are counted from the established reference point.

The success of sectioning vs. whole otolith mounts for mark detection suggests that sectioning is the better technique, especially for short-term studies. Because sectioning is time-intensive, use of whole mounts is more desirable for long-term or large-scale studies. When fish were at large for 30 days or more, whole mounts were successful at detecting AC marks 95% of the time. For many studies, the time saved by using whole mounts instead of sectioning may compensate for the 5% margin of error.

Conclusions

AC is an effective chemical for producing a single ring mark on otoliths from Mozambique tilapia using the methods described in this study. We only used 1 size of tilapia and a single AC concentration and duration, however, and results may differ

for larval or adult fish. Using higher concentrations of AC and immersion for longer periods may improve marking success and readability, and further study is warranted. In addition, mark duration was only evaluated for 60 days, and although mark visibility did not appear to diminish after that period, a longer period of validation would be necessary for stock assessment studies. However, due to the high cost of AC (about US$15/g) and due to FDA restrictions on chemical use in potential food fish, its use may be primarily restricted to small-scale validation studies in a controlled environment.

This chemical may also be useful at creating visible marks on otoliths of related cichlid species such as other tilapia and peacock bass (*Cichla* spp.), and pilot markings are suggested to evaluate marking success. We suggest a waiting period of at least 30 days before otoliths are sacrificed for mark examination, especially for studies using whole otolith examination techniques. The differential mark visibility between otolith preparation techniques advises caution for researchers using the whole otolith method, and further illustrates the importance of validation procedures.

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